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DETERMINATION OF INORGANIC SULFATE IN HUMAN SALIVA AND SWEAT BY CONTROLLED-FLOW ANION CHROMATOGRAPHY

NORMAL VALUES IN ADULT HUMANS*

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SUMMARY

Following the previous demonstration that low concentrations of inorganic sulfate (SO_4) in human serum and cerebrospinal fluid can be accurately determined by controlled-flow anion chromatography, the assay has been extended to the quantitation of free SO_4 in saliva and sweat by modification of the established methods of sample collection and preparation. Salivary secretions were ultrafiltered to remove macromolecular polyanions that bind irreversibly to the anion-exchange separator column and reduce resolution. Sweat was collected from 22 fasted adult volunteers using a method which utilizes absorbent filter pads applied to the forearm after secretion had been stimulated by pilocarpine iontophoresis. It was necessary to acid wash the filter pads to reduce sulfate contamination. Saliva ultrafiltrate or sweat was diluted and injected onto a Dionex D-10 Ion Analyzer using the standard anion column system.

The mean inorganic SO_4 concentration in saliva from seventeen adult fasting volunteers was 72 ± 4 μmol/l (± S.E.); the mean SO_4 concentration in sweat was 83 ± 3 μmol/l. Both are significantly less than in matching serum, suggesting that SO_4 is actively removed during formation of these glandular secretions. The ion chromatographic assay is shown to be capable of measuring SO_4 in biological fluids at concentrations that are otherwise undetectable by conventional assay techniques.

INTRODUCTION

The measurement of inorganic sulfate (SO_4) in complex matrices is significantly compromised by the relative insensitivity and non-specificity of

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classical chemical methods [1, 2]. In biological fluids, procedures that depend on selective barium sulfate precipitation suffer from incomplete reaction sequences if the SO_4 concentration is much less than 150 μmol/l [3, 4]. Sensitive precipitations of SO_4 with organic dyes, such as benzidine [5, 6], or precipitations of excess barium with chloranilate [7], or rhodizonate [8], are sufficiently non-specific that the physiological concentrations of co-attendant phosphate and chloride ions render assay results of unmodified fluids uninterpretable [1, 9]. Controlled-flow anion chromatography measures SO_4 by its conductimetric properties after it has been separated from other anions, and is ideally suited to the assay of biological samples [10—13].

Recent experiments have shown that the high sweat chloride, which is a hallmark of cystic fibrosis, arises from a defect in anion transport [14]. With this in mind, we set out to develop methods for measuring trace anions in sweat and saliva, focusing first on SO_4, which may be a useful analogue of chloride in the study of membrane transport [15, 16].

In an effort to extend the method for SO_4 determination to secretions of salivary and sweat glands, it was necessary to modify current procedures for sample collection and preparation before analysis. This report details the assay modifications and estimates of precision. Data are also presented for normal SO_4 concentrations in adult human secretions.

METHODS AND MATERIALS

Subjects

Adult laboratory personnel (mean age 26.2 years, range 18—43 years) gave informed consent to procedures for the collection of saliva, sweat and serum. Subjects were instructed to fast after midnight and to take only small amounts of tap water in the morning. Samples were collected between 09.00 and 12.00 h. If saliva was to be collected, subjects were also asked to forego brushing their teeth since many toothpastes contain sodium dodecyl sulfate. Experimental protocols were approved by the ethics committees of the I.W. Killam Hospital for Children and Dalhousie University Faculty of Medicine.

Sample collection and preparation

Blood

Blood was drawn from the antecubital vein using disposable needles and syringes and transferred to capped 25-ml Corning centrifuge tubes (Corning Glass Works, Corning, NY, U.S.A.). The blood was centrifuged and the serum supernatant stored at −20° C until assayed.

Saliva

Saliva was collected in disposable polystyrene cups, after subjects had thoroughly rinsed their mouths, gargled with distilled, deionized water (ddH_2O), and discarded the first few ml of saliva. Subjects chewed on pieces of Parafilm "M" wax (Marathon, Neenah, WI, U.S.A.) to stimulate flow but no effort was made to standardize or quantitate flow-rate. After 15 min, or when

a volume of 20—30 ml was obtained, the sample was centrifuged for 10 min at 1000 *g* to remove particulates and the supernatant stored at —20°C until assay.

Ultrafiltration of saliva. Injection of unfiltered saliva into the ion chromatograph results in significant loss of resolution. An Amicon Micropartition System (MPS-1, Amicon Corporation, Danvers, MA, U.S.A.) was therefore used to filter saliva samples. The MPS-1 system utilizes YMT cellulosic membrane filters that have a nominal molecular weight cut-off of 25,000, and generates an ultrafiltrate by centrifugation partition [17]. Integrity of the filters was tested by checking the ultrafiltrate for protein with urinary dipsticks (Labstix-R, Ames Company Division, Miles Labs., Rexdale, Canada).

In initial trials, it was noted that ultrafiltered saliva samples had more than twice the SO_4 content of the same samples analysed neat (Table I). The adoption of an acid-washing procedure of the membrane filters prior to processing the saliva was instituted to eliminate contamination. Washing the filters with four successive 1-ml aliquots of 1 m*M* hydrochloric acid reduced the concentration of SO_4 eluted from 57 to 5 μmol/l. (The SO_4 contamination of the hydrochloric acid before filtration was less than 1 μmol/l.) As Table I indicates, samples of saliva that are filtered with membranes washed as described above contain the same amount of SO_4 (71 ± 1 μ*M* vs. 70 ± 1 μ*M*, $n = 3$). This procedure was therefore adopted for all future studies. A saline blank was added in each run to ascertain that any particular batch of washed filters added less than 5 μmol/l SO_4 to the ultrafiltrate.

TABLE I

EFFECT OF ULTRAFILTRATION ON THE SULFATE CONTENT OF HUMAN SALIVA

Results are the mean ± S.E. ($n = 3$).

Sample	SO_4 Concentration (μmol/l)		
	Unfiltered	Filtered	
		Fresh filters	Acid-washed filters
Dilute hydrochloric acid (1 m*M*)	0.37 ± 0.09	58→17→15→5*	5**
Saliva	73 ± 2	171 ± 6***	—
	70 ± 1	—	71 ± 1§

*SO_4 Concentrations of successive 1-ml washes of the filter with 1 m*M* hydrochloric acid.
**If the filters contained more than 5 μmol/l, they were discarded.
***Significantly higher than the same sample before filtration ($p < 0.01$, Student's *t*-test).
§No significant effect if acid-washed filters are used.

Frozen samples of saliva were thawed and recentrifuged at 1000 *g* for 10 min to remove any futher precipitate. Approximately 1 ml of the supernatant was added to the MPS-1 apparatus. A 5-min run at 4°C and 1900 *g*, in an RC2-B Sorvall refrigerated centrifuge equipped with a fixed-angle SS-34 rotor (Sorvall, Newtown, CT, U.S.A.) was used to obtain an initial 100 μl of ultrafiltrate. Because the acid-washed membrane filters could not be adequately dried, the initial ultrafiltrate, diluted somewhat with wash fluid, was discarded. The

sample was then centrifuged for a further 30 min at 1900 *g* and the final 200—300 μl of ultrafiltrate were collected, diluted fifteen-fold with 1 m*M* sodium hydroxide, and analyzed immediately.

Sweat

The collection of sweat on absorbent discs is a standard clinical procedure for measurement of sweat chloride in the diagnosis of cystic fibrosis. A modification of the method of Gibson and Cooke [18, 19] was used, as outlined below.

Preparation of absorbent discs. Cellulose filter discs [25 mm diameter, Type 470 (Lot F25), Schleicher and Schnell, Keene, NH, U.S.A.] also contained significant amounts of SO_4. To remove this contamination, discs were placed in Fisherbrand-R Histological Processing Cassettes (Product 12-653-16A, Fisher Scientific, Dartmouth, Canada), rinsed in three successive solutions of 5 m*M* nitric acid (prepared from reagent-grade nitric acid; J.T. Baker, Phillipsburg, NJ, U.S.A.), and finally in ddH_2O. Drying was facilitated by brief rinses with 95% (v/v) ethanol and acetone. Discs were placed in 25-ml polypropylene weighing bottles (Markson Science, Del Mar, CA, U.S.A.) and stored in a desiccating chamber until used. Under these conditions, the disc dry weight remained constant over 24 h.

Collection. Both ventral forearms were wiped with low-sulfate gauze (Curity Gauze Sponges, 12-ply, 10 × 10 cm; Kendall Canada, Toronto, Canada) that had been dampened with ddH_2O. Pilocarpine hydrochloride (1%, w/v; Sigma, St. Louis, MO, U.S.A.) and sodium nitrate (2%, w/v) were used to soak gauze pads attached to the ventral anodic and dorsal cathodic plates, respectively. The padded plates were applied to each forearm, and the current was maintained at 1.5 μA for 5 min, after which the iontophoresed areas were again wiped clean with dampened gauze, then dried.

Two absorbent filter discs, prepared as described above, were removed from their weighing bottles and placed under a Parafilm covering that was then carefully taped to the arm. After 45 min, the sweat-laden discs were returned to the weighing bottle and the total weight was recorded. Subtraction of the dry weight (in the bottle) yielded the weight of collected sweat and hence its volume. (The density of sweat of any collection in this manner is sufficiently close to that of water that any correction adds less than 1% to the overall accuracy [20].)

If the weight of sweat collected was less than 50 mg, it was discarded; when it was less than 100 mg, it was pooled with the matching sample from the opposite forearm. Exactly 4.00 ml of 1.0 m*M* sodium hydroxide were added to the weighing bottles containing the discs. Sweat was eluted into the sodium hydroxide solution over at least 10 min at room temperature, with occasional agitation of the discs in the solution. The sodium hydroxide eluate was then decanted and spun at 1000 *g* for 5 min to remove any cellulose fibres loosened from the discs. An aliquot of the supernatant was immediately subjected to chromatographic analysis.

Recovery studies

Saliva

Increasing amounts of 50 m*M* sodium sulfate were added to aliquots of a pooled saliva sample, which were then filtered and analyzed by the methods described above.

Sweat

Sweat samples were large enough for analysis, but recovery studies cannot be performed on samples obtained by the iontophoretic method using absorbent discs. Instead, tracer sulfate ($^{35}SO_4$, as carrier-free sulfuric acid; New England Nuclear, Montreal, Canada) was added to prepared dry discs or to discs soaked with varying amounts of ddH_2O or Ringer's lactate. The radioactive sample was then eluted in the standard fashion. The eluate was counted to determine whether any of the added SO_4 remained bound to the disc. Similarly, varying amounts of 100 μM sodium sulfate were added to blank discs and the samples prepared and analyzed in the standard fashion.

Binding studies

Both serum and saliva contain proteins and glycoproteins that are known to bind multivalent ions. To determine whether a significant fraction of sulfate is also significantly bound, small amounts of labelled SO_4 were added to serum, saliva, and saline solutions and subjected to ultrafiltration. The specific activity of the ultrafiltrate was then compared to that of the unfiltered sample.

ANALYTICAL

Controlled-flow anion chromatography

All samples were analyzed on a D-10 ion chromatograph (Dionex, Sunnyvale, CA, U.S.A.). For SO_4 analysis, this instrument was outfitted with two 50 X 4 mm guard columns (containing the same anion-exchange resin as the main separator column), a 250 X 4 mm separator column (AS1, standard anion analysis) and a fixed-bed, 100 X 9 mm S1 suppressor column in series. To reduce the leaching of metal ions from the plumbing into the cation-exchange resin of the anion suppressor column, a cation trap was added to the regenerating system. With biological samples, it was found that the suppressor must be regularly exhausted with 0.1 *M* sodium carbonate, thoroughly rinsed with ddH_2O, and vigorously regenerated with 1 *M* sulfuric acid, if the eluate is to remain free of contaminants and a stable baseline is to be maintained. After approximately 200 samples had been processed, the first pre-column was discarded, the second pre-column moved forward to the position of the first, and the second pre-column replaced with a new one.

Sulfate concentrations were calculated by interpolation using the line of best fit for peak heights of at least four standards. All standard curves used in this study had correlation coefficients greater than 0.99.

Liquid scintillation counting

Aliquots (20 μl) of samples (serum, sweat, saliva, etc.) containing radio-

labelled SO_4 were transferred to mini-vials containing 4 ml of Readi-Solv scintillating fluid (Beckman Instruments, Fullerton, CA, U.S.A.) and counted by liquid scintillation.

Reagents

All chemicals were reagent grade unless otherwise indicated. Solutions were tested by ion chromatography and all contained less than 1 μmol/l SO_4. Because there is a tendency for the sodium hydroxide reagents and solutions to become contaminated with ambient SO_4, fresh 1 m*M* solutions were prepared monthly from individually preweighed vials of pelleted sodium hydroxide (product No. 505-8, Sigma). These solutions had the lowest SO_4 content of the several commercially available preparations assayed. All distilled, deionized water used had a resistance of greater than 10 MΩ and contained no detectable SO_4, as measured by ion chromatography (limit of sensitivity < 0.1 μmol/l).

Statistical analysis

Statistical analyses were performed according to the methods described by Sokal and Rohlf [21]. Like many biological variables, the distribution of SO_4 concentrations often displays upward skewing; where this might distort the statistical interpretation, data were log-transformed prior to analysis.

RESULTS

Chromatographic profiles

Sulfate in saliva emerges as a symmetric peak at 17.0 min (Fig. 1). It is well separated from other anions and readily quantitated by peak height. Sulfate in sweat elutes at 16.3 min as a less symmetric peak that often exhibits tailing (Fig. 1). However, a horizontal baseline can be drawn and the sweat SO_4 measured by peak height as for other samples. Earlier peaks (not labelled) shown in Fig. 1 may be nitrate and bromide [12], but a positive identification was not attempted. Other unidentified substances, indicated by a question mark, were occasionally observed.

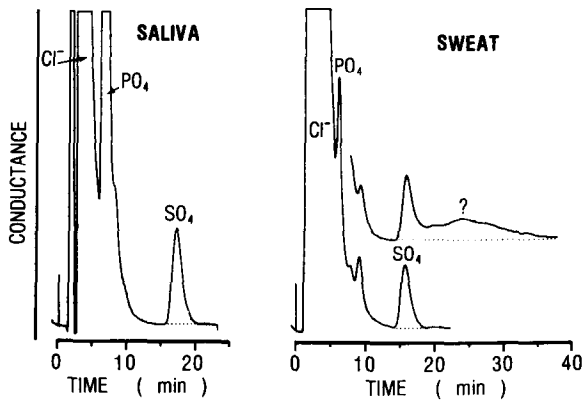

Fig. 1. Chromatographic profiles for saliva and sweat. Scale on the abscissa is arbitrary, based on a 1 μS full-scale deflection and a fixed baseline offset for background conductivity. Baselines for SO_4 determinations are shown as dotted lines.

Recovery studies

As Fig. 2 shows, there is good linear correlation between measured SO_4 and the SO_4 added to saliva prior to ultrafiltration and analysis. The line of best fit ($y = 0.89x + 9.0$; $F_{1,9} = 14.2$, $p < 0.01$) has a slope not significantly different from 1.0 (95% confidence limits: 0.67—1.07). Mean recovery was 96.1 ± 1.4% (mean ± S.E., $n = 10$).

Recovery of radiolabelled SO_4 added to the prepared sweat discs was significantly affected by the ionic strength of solution. Free radiolabelled SO_4 in ddH_2O discs was not completely eluted when fluid volumes of less than 100 µl were added to dry discs; the same radiolabel added to discs soaked in small volumes (< 100 µl) of Ringer's lactate was readily eluted with a recovery of 100 ± 7% (Table II).

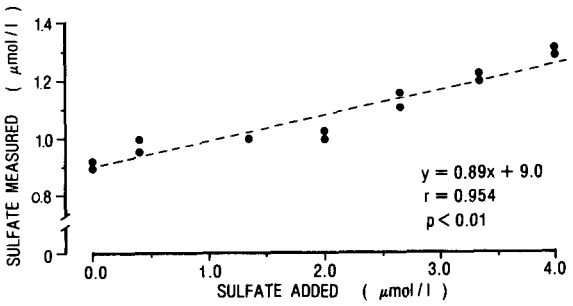

Fig. 2. Recovery of SO_4 added to filtered saliva. Line of best fit, by the least-squares method, is shown; its slope does not differ significantly from 1.00.

TABLE II

RECOVERY OF SO_4 FROM ABSORBENT FILTER DISCS USED IN SWEAT COLLECTION

Addition	*n*	Amount added	Amount recovered	Recovery (%)
Radiolabelled SO_4 (cpm)				
Discs alone	6	671 ± 11*	739 ± 57	110 ± 9
Discs with sweat	2		682 ± 82	102 ± 12
Unlabelled SO_4 (µmol/l)				
Dry discs: < 100 µl	9	100	82 ± 7	82 ± 7
> 100 µl	4	100	105 ± 3	105 ± 3
Discs with Ringer's lactate (50—100 µl)	4	123 ± 4**	123 ± 9	100 ± 7

*Mean ± S.D. of six aliquots.

**Mean ± S.D. of four measurements of Ringer's lactate to which was added a small amount of 50 m*M* sodium sulfate, equivalent to 100 µ*M* in final dilution.

Binding studies

The specific activities of radiolabelled SO_4 were the same in the ultrafiltrates of both serum and saliva as in the original fluids (Table III). Thus, there is no evidence of significant binding of SO_4 to macromolecules, nor to the ultrafiltration membranes themselves.

TABLE III

EFFECT OF MPS-1 ULTRAFILTRATION ON RADIOLABELLED SO_4 IN HYDROCHLORIC ACID, SERUM AND SALIVA

Mean ± S.E. of cpm for three determinations are shown.

Sample	Before ultrafiltration	After ultrafiltration	*p*
1 m*M* Hydrochloric acid	2180 ± 15	2165 ± 20	NS*
Saliva	2080 ± 20	2125 ± 10	NS
Serum	2104 ± 27	2387 ± 16	< 0.05**

*NS, Not significant.

**Specific activity of the ultrafiltrate is marginally but significantly higher than the unfiltered serum. This probably represents the concentrating effect of protein exclusion by ultrafiltration, which amounts to about 7% [32].

Estimates of precision

Within-run coefficient of variance for pooled saliva samples was 4.2% (n = 9). Between-run coefficient of variance was 2.7% (n = 22).

Duplicate samples of sweat from the same individual yielded a maximum estimate for method variability (Fig. 3). Two-way analysis of variance on eighteen duplicates, one from each forearm, yielded a within-patient variability that was 9.9% of the total, of which only a portion can be methodological in origin.

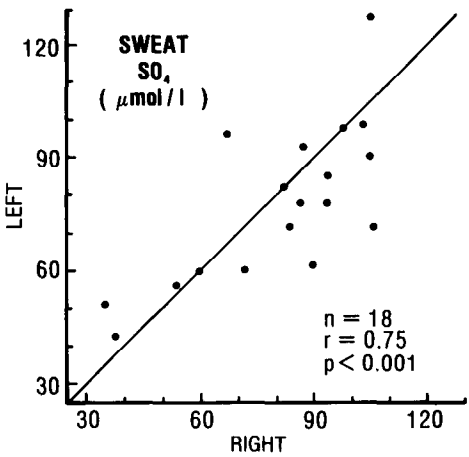

Fig. 3. Correlation between sweat SO_4 concentrations simultaneously obtained from left and right forearms of each subject. The line of identity is shown and with it the correlation coefficient.

SO_4 Concentrations in saliva

The mean concentration of SO_4 in saliva from seventeen adult fasted volunteers was 72 μmol/l (range 52—107 μmol/l) (Fig. 4A). The matching serum concentration was 298 ± 9 μmol/l, which is comparable to values reported previously [11]. The difference between serum and saliva concentra-

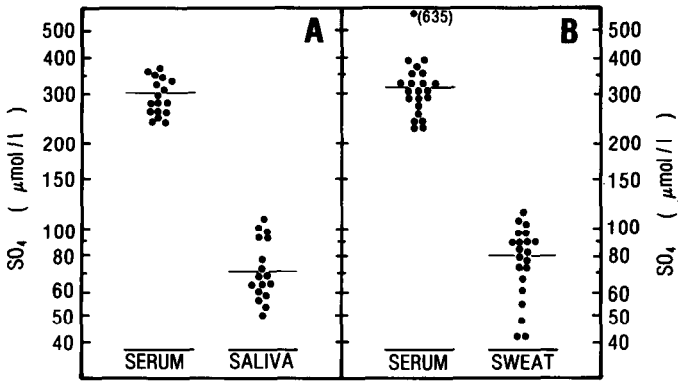

Fig. 4. SO_4 Concentrations in saliva (A) and sweat (B). Note the logarithmic scale of the ordinate. Untransformed means are shown as horizontal lines. Both saliva and sweat SO_4 concentrations are significantly lower ($p < 0.001$) than in matched sera.

tions is highly significant ($p < 0.001$, Student's t-test). There were no significant differences between mean SO_4 concentrations in the ten female (71 ± 6 µmol/l) and seven male (74 ± 7 µmol/l) volunteers. Mean salivary SO_4 concentration was about one-half of that reported previously for cerebrospinal fluid [135 ± 14 µmol/l (mean ± S.E., $n = 25$), $p < 0.001$; Student's t-test] [13].

SO_4 Concentrations in sweat

Mean weight of sweat was 222 ± 16 mg (range 87—431 mg). For sample duplicates ($n = 18$), values for right and left forearms were significantly correlated ($r = 0.75$, $p < 0.01$) (Fig. 3). There was no significant difference in mean sweat weight or SO_4 concentrations between the duplicates (data not shown); therefore, when the sample volume was small, duplicates were pooled for analysis.

Sulfate concentration in sweat from 22 volunteers was 80 ± 3 µmol/l ($n = 39$; range 35—128 µmol/l), while the matching serum concentration was 299 ± 25 µmol/l (Fig. 4B). Serum SO_4 in males and females did not differ significant-

TABLE IV

SULFATE CONCENTRATIONS IN HUMAN SALIVA AND SWEAT

All values given are mean ± S.E. µmol/l.

Group	No. of individuals	Saliva	Serum	No. of individuals	Sweat*		Serum
					Weight (mg)	SO_4**	
Females	10	71 ± 6	287 ± 13	9	221 ± 26	79 ± 5	316 ± 55
Males	7	74 ± 7	306 ± 13	13	531 ± 55	81 ± 7	287 ± 19
Combined	17	72 ± 4	298 ± 9	22	404 ± 47	81 ± 4	299 ± 25

*Total sweat weight from both forearm collections is given.

**SO_4 Concentrations used in this calculation are means of both forearm collections in all but four cases where samples were combined to yield adequate volume for analysis.

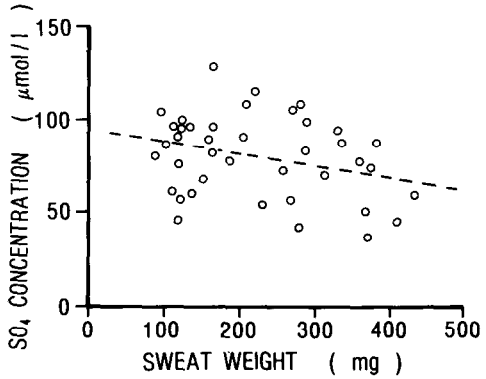

Fig. 5. Dependence of SO_4 concentration on sweat weight. Line of best fit obtained by least-squares regression and analysis of variance is also shown. Note that the influence of sweat weight is modest when compared to the overall variation in SO_4 concentrations.

ly in this study (Table IV) and, although the combined sweat weight in males was more than twice that in females, there was no sex-related difference in sweat SO_4 concentrations.

The possible influence of sweat weight, a measure of secretion rate, on sweat SO_4 concentrations was also examined (Fig. 5). The correlation coefficient was -0.32 ($p = 0.0025$). Analysis of variance for a linear regression model yielded an F statistic ($F_{1,38} = 4.195$) that was just below the 5% level of significance ($p = 0.048$); continued higher-order polynomials did not significantly improve the fit. The slope of the line ($y = -0.067x + 94.8$) is such that sweat weight exerts little influence on normal values.

DISCUSSION

The ultimate composition of the fluids produced by compound exocrine glands is a reflection of two interacting transport processes. The first is initial fluid formation; the second, subsequent modification of fluid by selective reabsorption [22, 23]. Current evidence suggests that the primary secretion is isotonic with extracellular fluid and is equivalent to serum ultrafiltrate [22, 24]. Sweat and salivary glands are no exception, although they differ significantly in fine structure and function. Determination of the solute profiles in human subjects presents a technical challenge for all but the most abundant components. While it is apparent that excretion of electrolytes in sweat or saliva plays only a secondary role in mineral homeostasis [25], fluid composition may provide important clues to the nature of systemic electrolyte transport disorders. Such is the case in cystic fibrosis [26].

Previously, we have shown that controlled-flow anion chromatography is a specific, sensitive and precise method for SO_4 determinations in serum and cerebrospinal fluid [11, 13]. In this report, the same is shown for sweat and saliva. To eliminate the problems of SO_4 contamination, the standard collection procedures had to be modified. In doing so, normal values for saliva and sweat SO_4 in fasting adults have been derived for the first time.

Previous attempts to estimate SO_4 concentrations in human secretions have been made, but total sulfur content [27], or an upper limit [25], are the only values reported. For saliva, the usual procedures which depend on precipitation

of barium or benzidine fail, presumably because these cations bind significantly to strongly charged polyanions (collectively termed "mucus") found in the final salivary product [28]. We found that we could measure SO_4 in the presence of these polyanions, but only at the expense of column degradation and eventual loss of resolution. Ultrafiltered samples, however, were assayed without difficulty. As yet, no attempt has been made to assess the effects of circadian variation [29], gland stimulation, or altered secretion rate on saliva SO_4 concentrations. Moreover, there are significant compositional differences among the fluids of different salivary gland groups which cannot be assessed using whole saliva [30] and would require a more elaborate collection technique.

Reliable collection of human sweat requires attention to technique. This has been amply demonstrated for chloride estimations [20] and proved to be extremely important for SO_4, which is present in a molar abundance that is less than 0.4% of that for chloride. It is fortunate that little manipulation of the sweat sample is required prior to analysis. However, rigorous attention to the elimination of artifacts is still required. As with salivary secretions, we did not attempt to estimate the effects of circadian variation or physiological stimuli on sweat SO_4 concentrations.

Pilocarpine iontophoresis produces a maximum stimulatory response that presumably allows for meaningful comparisons between controls and patient populations [20]. We found a small but significant bias toward lower values at high sweat rates, but the magnitude of the bias was not so large that a corrective factor is required.

It was not surprising to find that SO_4 concentrations are low in both sweat and saliva. The reabsorptive properties of the ductal epithelium are such that the potential difference across the epithelium is highly negative with respect to the lumen. Thus, there is a significant electrical driving force for the transport of anions out of the lumen, across the luminal membrane, and into the epithelial cytosol. From there, the anion diffuses through the cytosol, is transported across the apical membrane, and re-enters the extracellular or intravascular pool [22]. The origin of the potential difference is thought to be the pumping of Na^+ by Na^+—K^+-ATPase across the apical membrane and out of the epithelial cell, followed by influx of cation from the lumen down its chemical gradient into the cell. The passive influx of cation from the lumen thus generates a negative potential in the luminal space.

Chloride reabsorption can thus be viewed as following the pathway of Na^+ absorption into the ductal epithelium, then transcellular diffusion and uphill efflux. Whether the same is true of SO_4 cannot be ascertained from the present data. However, it seems likely that the lower SO_4 concentrations of the final secretion are a reflection of selective ductal reabsorption.

This report illustrates that trace electrolytes can be accurately measured by controlled-flow ion chromatography in very small samples of secreted fluids. It will be of more general interest to determine whether this method can be adapted to generate anion or cation profiles for different secretions. With the appropriate technical modifications, it should be possible to measure chloride, bicarbonate, lactate, phosphate and sulfate simultaneously [10, 31]. Application of such a method might well be very rewarding in the effort to identify the anion transport defect that characterizes the sweat duct in cystic fibrosis [22].

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